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Factors Affecting the Growth of Staphylococcus Aureus Strains Capable of Producing Enterotoxins A, B, C and D in Sterile Milk

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FACTORS AFFECTING THE GROWTH OF *STAPHYLOCOCCUS AUREUS* STRAINS CAPABLE OF PRODUCING ENTEROTOXINS A, B, C AND D IN STERILE MILK

by

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Manoj A. Divatia

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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by

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Growth and enterotoxin production by Staphylococcus aureus strains, in the presence of different starter [2:1 (V/V) blend of AM_2:ML_8 strains of Streptococcus lactis] levels was investigated. Sterile, 10 per cent non-fat dry milk was inoculated with S. aureus strains capable of producing all four types of enterotoxins, and reduced levels of starters; and was incubated at 32 C for 24 hours. The pH and S. aureus population were determined at 2 hour intervals until 8 hours and at 24 hours. The inhibitory response of lactic streptococci was studied by spot-tests on a lawn of S. aureus strains.

The drop in pH, from 4 to 8 hours incubation, for all starter levels, was proportional to their inocula. The rate of acid formation, or drop in pH, from 4 to 8 hours, was correlated with the change in staphylococcal population from 6 to 24 hours (Correlation Coefficient = $\gamma = -0.805$). Regression analysis indicated that change in pH from 4 to 8 hours

could be used to predict the staphylococcal population change from 6 to 24 hours.

All four enterotoxigenic strains showed differential susceptibility to the starter metabolite(s). A 0.1 per cent starter level did not allow the increase of approximately 10^4 cells per milliliter, of an enterotoxin D producing strain of S. aureus (23235). Approximately 10^6 cells per milliliter of S. aureus 23235, decreased to about 10^4 cells in the presence of a 0.1 per cent starter level; while 0.01 per cent starter level did not allow the inocula of approximately 10^2 cells per milliliter to increase. The inocula of approximately 10^6 cells per milliliter, of enterotoxin B producing strain of S. aureus, did not increase in the presence of 0.01 per cent starter level. The same inocula of enterotoxin A and C producing strains of S. aureus decreased to about 10^3 to 10^4 cells per milliliter in the presence of 0.01 per cent starter. These strains sharply declined in population in the presence of 0.1 per cent starter level.

The lactic organism did not produce inhibitory levels nisin, or over 5 micrograms of hydrogen peroxide per milliliter of broth. When the lactic streptococci were spotted on lawns of enterotoxins B, C and D producing strains of S. aureus, staphylococcal growth was inhibited around the spots, on both agar, with and without added calcium carbonate. Enterotoxin A producing strain was not inhibited on any agar. The degree of inhibition for B and D enterotoxin

producing strains, was greater in agar fortified with calcium carbonate, than that without fortification while the reverse was true for enterotoxin C producing strain.

(69 pages)

INTRODUCTION

The National Communicable Disease Center, Atlanta, Georgia, has reported that staphylococcal food poisoning outbreaks ranged from 23.8 to 27.5 per cent of total outbreaks reported from 1967 to 1970. The patients involved varied from 8.7 to 25.2 per cent of the total (National Communicable Disease Center 1967, 1968, 1969, 1970). Staphylococcal food poisoning, therefore, is still of significant public health concern. Pasteurized milk cheese produced under modern conditions has been implicated in staphylococcal poisoning outbreaks even though proper milk-storage temperatures and heat treatments should have minimized the public health menace (Zehren and Zehren, 1968b).

It has been recognized that Staphylococcus aureus is one of the principal etiological agents of bovine mastitis (Janzen, 1970). In an attempt to explore the relationship between staphylococcal food poisoning and bovine mastitis, it was observed that the infected bovine mammary gland represented a significant reservoir of enterotoxigenic strains of S. aureus (Olson et al., 1970).

Several workers evaluated the population changes and enterotoxin production by S. aureus in milk and dairy products during manufacturing (Donnelly, Leslie and Black, 1968; Reiter et al., 1964; and Tatini et al., 1971). It has been accepted that starter culture growth and activity in fermented

dairy products has an inhibitory effect on S. aureus (Jezeski et al., 1967; Reiter et al., 1964). Studies concerning the minimum activity of starter cultures required to inhibit the growth of S. aureus have not been reported. It has been recognized that following a bacteriophage attack there would be some lactic acid organisms remaining active and capable of acid production (Collins, 1962). Since the severity of bacteriophage infestation varies greatly and the ability to reproduce the same bacteriophage effect is difficult, reduced levels of starter inocula were used in this study. The lactic acid organisms used in this study reflect the current practices in New Zealand and Australia (Breen, 1969). Combination of the two strains utilized in this study, consistently produced bitter-free, and good flavored cheddar cheese.

In order to obtain useful information in establishing a plausible control measure for the staphylococcal intoxications through dairy products, an investigation was carried out to study the growth ability of staphylococci in the presence of reduced levels of the starter bacteria.

REVIEW OF LITERATURE

Detection Methodology and Properties of Enterotoxins

Few attempts were made in the period from 1931 to 1950 to ascertain the nature of the staphylococcal enterotoxin. Investigators disagreed as to whether the enterotoxin was a carbohydrate or a protein (Minnet, 1938; Davison, 1940; Hammon, 1941). In 1948, a long-range investigation was undertaken at the Food Research Institute, then associated with the University of Chicago, to elucidate the properties of the enterotoxin. In the preliminary study it was assumed that the causative agent of staphylococcal intoxication was a single substance. After the purification of a protein which could induce illness in monkeys, the existence of more than one enterotoxin was discovered (Bergdoll, 1967).

The major problem confronting investigators was the lack of a practical quantitative assay. Several species of animals, including cats and dogs, (Dolman, Wilson, and Cockcroft, 1936), monkeys (Jordan and McBrown, 1931; Surgalla, Bergdoll and Dack, 1953); frogs (Eddy, 1951, Robinton, 1949), and pigs (Hopkins and Poland, 1942) have been used to determine biological activity of enterotoxins. Animals other than monkeys are relatively insensitive to enterotoxins unless they are injected intraparitoneally (I.P.) or intravenously (I.V.). The methods employed most

frequently are I.P. or I.V. injections of cats and kittens (Dolman, Wilson and Cockcroft, 1936; Hammon, 1941) and feeding of young rhesus monkeys (Surgalla, Bergdoll and Dack, 1953). It is necessary to inactivate substances that may provoke symptoms similar to those caused by enterotoxin when administered by parenteral routes if the cat method is used. Heating at 100 C for 20 to 30 min (Davison, Dack, and Cary, 1938; Dolman, 1936), treatment with trypsin (Denny and Bohrer, 1963), and use of antisera (Dolman, 1943) to inactivate the interfering substances have been used with success. However, the assurance of complete inactivation of all interfering substances is not easy. Another disadvantage is that cats are relatively insensitive to enterotoxin C, and require approximately 50 times more enterotoxin C than A or B (Casman, Bennett, and Kephart, 1966).

Dolman and Wilson (1938) reported a specific antibody for the staphylococcal enterotoxin and suggested that the problem of enterotoxin detection might be solved by serological methods. In 1947, the Division of Microbiology, Food and Drug Administraion, Washington D.C., initiated a long-range program to develop serological methods for enterotoxin detection. Casman (1958, 1960) demonstrated: (a) the antigenicity of enterotoxin by conferring passive immunity to cats by the antiserum produced in rabbits; (b) the occurrence of two serological types of enterotoxins; and (c) an immunodiffusion test in gel for detection of enterotoxins. This

test facilitated detection of enterotoxin from foods (Casman, Bergdoll, and Robinson, 1963). The immunological reaction may not necessarily indicate biological activity; however, in most instances correlation between the two is adequate to justify using the immunological reaction in assaying enterotoxins. This method was used by Casman, Bergdoll and Robinson, (1963) to identify and designate the enterotoxins as A, B and C.

To date, four enterotoxins have been identified (Bergdoll, 1969; Casman et al., 1967; Casman, 1969). In the course of investigations at the Food Research Institute, two enterotoxin "C"s were purified from two different strains of S. aureus. Enterotoxin C of strain 137 has an isoelectric point of 8.6 and is referred to as C_1; while that of strain 361 has an isoelectric point of 7.0 and is referred to as C_2 (Bergdoll, 1967).

A study to understand the toxicological properties of enterotoxin by modification of chemical groups on enterotoxins has been reported (Borja and Bergdoll, 1969). These and other studies have produced information on biochemical and biophysical properties of enterotoxins, which is summarized in Table 1.

Significant progress has been made in the methodology for detection of staphylococcal enterotoxins. Development of immunodiffusion techniques for the quantitative detection of enterotoxin marked a significant improvement over the animal tests. Gandhi and Richardson (1971) described an

Table 1. Biophysical and biochemical properties of enterotoxins.

	Enterotoxin identity				
	A[a]	B[b]	C_1[c]	C_2[c]	D[d]
Molecular weight	34,700	35,300	34,100	34,000	--
Partial specific volume	0.726	0.743	0.732	0.742	--
Nitrogen content (%)	16.5	16.1	16.2	16.0	--
Sedimentaion coefficient	3.04	2.89	3.00	2.90	--
Diffusion coefficient (D^{o}_{20}, W) x 10^{-7} cm^2/sec.	7.94	7.72	8.10	8.10	--
Reduced viscosity (ml/gm)	4.07	3.92	3.4	3.7	--
Isoelectric point	6.8	8.6	8.6	7.0	--
Maximum absorption (nm)	277	277	277	277	277
Extinction ($E^{1\%}_{1\ cm}$)	14.3	14.0	12.1	12.1	--
N-terminal amino acid	Alanine	Glutamic acid	Glutamic acid	Glycine	--
C-terminal amino acid	Serine	Lysine	Glutamic acid	Glycine	--
Emetic dose (ED_{50}) (Monkey) µg/animal	5	5	5	5-10	1.5 (cats)
Maximum percent purity obtained	99.1	99.0	99.7	99.8	40

[a]Chu et al., 1966.

[b]Schantz et al., 1965.

[c]Bergdoll, Borja and Avena, 1965.

[d]Casman et al., 1967.

immunodiffusion of liquid reactants in capillary tube. The diameter of these tubes was 1.0 mm and they observed the ring formation at interface within 20 to 40 min. They indicated that 1 µg of enterotoxin/ml was detected and their method used very small quantities of reactants. It was proposed that this technique could be used as a quick screening test for suspected samples.

The single diffusion tube test (Oudin test) has been quite useful for detection and quantitation of enterotoxins (Hall, Angelotti, and Lewis, 1963; Weirether et al., 1966). The sensitivity of the Oudin test is approximately 1 µg of enterotoxin/ml.

The double diffusion technique (Oakley's test) can detect even 0.1 µg of enterotoxin/ml (Hall, Angelotti, and Lewis, 1965). The microslide modification of Ouchterlony's test (Crowle, 1958) was successfully applied to enterotoxin detection by Casman and Bennett (1963). The technique, though difficult to perform, has the advantage of determining the relationship between various enterotoxigenic cultures and culture fluids. Casman et al. (1969), after improving their method, claimed a sensitivity of 0.0625 µg of highly purified enterotoxin A and B/ml.

Immunofluorescence has also been applied for detection of enterotoxin B in cells, culture-media, and foods (Friedman and White, 1965; Genigeorgis and Sadler, 1966a, 1966b). An indirect hemagglutination inhibition procedure has been developed (Brown and Brown, 1965; Robinson and Thatcher,

1965). A floatation system reported by Hooper (1963) can detect concentrations of 1 µg of enterotoxin B/ml in 2 to 3 hr. The indirect hemagglutination inhibition method has problems associated with the presence of potent hemagglutinins for sheep erythrocytes in the staphylococcal cultures examined. Recently, Silverman, Knott, and Howard (1968) reported a reversed, passive hemagglutination assay. They claimed a sensitivity of 0.0007 µg of enterotoxin and the test could be completed in a few hours. This method required a considerable quantity of highly purified antibody, to satisfy the needs of antienterotoxic globulin. Microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B was described by Morse and Mah (1967). They claimed that with their technique many samples could be assayed simultaneously and results could be obtained in 3 hr. The small quantity of the reactants were required and they claimed that even 0.01 µg of toxin/well or 0.4 µg of toxin/ml could be detected. This method still has the problems associated with the presence of potent hemagglutinins for sheep erythrocytes in staphylococcal culture fluids and suspected foods. The sensitivity of the assays and time taken for completion of tests are summarized in Table 2.

Staphylococcal Growth in Milk and Incidences of Poisoning

The first milk-borne out-break of staphylococcal food poisoning was reported in the Philippines due to consumption

Table 2. Comparison of serological methods for enterotoxin assay

	Method	Reference	Sensitivity µg/ml	Type of enterotoxin	Remarks[a]
1.	Microslide gel double diffusion	Casman et al., 1969	0.0625	A, B; applicable to C & D	1) 3 days at room temp. 2) +
2.	Passive hemaglutination	Silverman et al., 1968	0.0007	A, B	1) Few hours 2) +
3.	Fluorescent antibody	Genigeorgis & Sadler, 1966a, 1966b	0.5 to 1.0	B	1) 4-6 hr 2) -
4.	Oudin's tech.	Weirether et al., 1966	2.0	B	1) 20-24 hr 2) +
5.	Hemaglutination inhibition	Morse & Mah, 1967	0.4 0.01 µg/well	B	1) 3-4 hr 2) -
6.	Capillary tube method	Gandhi & Richardson 1971	1.0	A, B, & D	1) 20 to 40 min 2) +

[a] 1) Time required for enterotoxin detection.
2) + (concentration of enterotoxin from food samples necessary).
- (concentration of enterotoxin from food samples not necessary).

of raw milk from an apparently healthy cow (Barber, 1914). Dauer (1961) traced five cases of staphylococcal food poisoning to consumption of unpasteurized milk. An outbreak of staphylococcal food poisoning from improperly cooled raw milk was reported by Steed, Buxton and Iredale (1962). They isolated S. aureus from 9 out of 32 cows in the herd. Food poisoning outbreaks caused by consumption of raw milk have been traced to Salmonella typhimurium and S. aureus (Vernon, 1965). Worseck and Hemlep (1960) isolated 650 strains of staphylococci from 22 raw milk samples, from which 16.7% were plasma coagulase positive. The toxinogenicity of seven of these strains were confirmed by kitten test.

Nilsson and Segerfeldt (1964) reported detection of coagulase positive staphylococci from 159 samples of raw, pasteurized, and mastitic milk samples. They noted that incidence was higher in mastitic milk samples. Anderson (1965) reported in a study conducted on incoming raw milk samples and processed milk products at a Swedish factory; that coagulase positive staphylococci were invariably found in raw milk. None of the pasteurized milk samples contained these organisms.

Allison (1949) considered 5×10^5 coagulase positive staphylococci/ml as the minimum viable population necessary to cause food poisoning. This conclusion was drawn from the results of kitten tests and viable counts of staphylococci. Occurrence of coagulase positive staphylococci in market milk in India was reported by Gehlot and Saraswat (1969).

In a survey of milk and milk products in India, Ghosh, Nambudripad, and Laxminarayana (1970) reported that 32.5% of the samples contained S. aureus, and out of 320 isolates 29 produced enterotoxin type A and B.

Smith (1957) observed poor growth of S. aureus in raw milk samples compared to heated milk. He indicated that the initial ratio of S. aureus population to that of the other organisms in raw milk had a profound effect on the growth of S. aureus. When this ratio was 2:1, the S. aureus populations reached 1.75×10^8/ml, at 37 C in 24 hr. Upon adjustment of this ratio to 1:5, S. aureus population reached 1.5×10^7/ml and became 3×10^6/ml when the ratio was adjusted to 1:50. Similar results were reported by Heinemann (1957) with S. aureus 161 and S_6 in raw milk at 21.1, 26.7, and 35 C. Micro-organisms of raw milk have been known to inhibit the growth of S. aureus, and Takahashi and Jones (1959) noted that the extent of this inhibition was directly proportional to the initial bacterial population of the milk. Thatcher and Ross (1960) indicated that extensive growth could occur under simulated conditions of improper milk cooling. They could not observe S. aureus growth in milk samples incubated at 10 C for 24 hr. Staphylococcus aureus, according to Clark and Nelson (1961), did not grow at 4 C, while it grew at 10 C after 7 days.

Reiter et al., (1964) investigated growth of S. aureus 30 in raw milk, in milk heated at 161 F for 17 sec, and in milk steamed at 212 F for 30 min for three consecutive days,

in presence and absence of 1% starter (Streptococcus lactis ML_3). They showed that S. aureus 30 increased 70 times in steamed milk, but less than twice in raw milk or pasteurized milk. This confirmed the presence of a heat labile inhibitor which was not affected by pasteurization temperature. In the presence of starter in steamed milk, the increase of staphylococci was only three and one-half times. The controlled increase in the presence of starter in steamed milk was not only due to lactic acid production but was also due to unidentified factor(s) produced by the starter. These workers did not succeed in characterizing the inhibitor(s). The initial population of S. aureus was approximately 1.2×10^4 to 4.5×10^4/ml. Production of enterotoxin A by S. aureus MF 224 in milk was studied by Donnelly et al., (1968). They reported the time required for enterotoxin A production by 10^6 S. aureus cells/ml in low count raw and pasteurized milk. Toxin was detected within 6 to 9 hr at 35 C, 9 to 12 hr at 30 C, 18 hr at 25 C and 30 hr at 20 C. When the inoculum was reduced to 10^4 S. aureus cells/ml, S. aureus growth and enterotoxin production took 50% more time than that taken by 10^6 S. aureus cells/ml inoculum at each respective temperature. They observed that toxic samples always contained 5×10^7 or more cells/ml.

The influence of S. lactis on growth of S. aureus and enterotoxin A production was reported by Jezeski et al., (1967). They observed that an enterotoxigenic strain of S. aureus was inhibited by an actively growing S. lactis

strain upon inoculation in sterile, reconstituted non fat dry milk (10% NDM) at 32 C. They studied growth inhibition for 72 hr and reported enterotoxin A detection in the 24, 36, and 72 hr milk samples with S. aureus alone, and with S. aureus, S. lactis and homologous lactic phage. They could not detect enterotoxin A in similarly incubated samples phage free with S. lactis and S. aureus. They concluded that the S. lactis and S. aureus ratio in inoculum was an important factor in determining S. aureus growth and enterotoxin production by S. aureus. They did not indicate the ratio which would be safe for industry. Minore and Marth (1970) reported a 99% reduction in population of S. aureus 100, at 37 C, over a period of 12 hr, in artificially acidified pasteurized milks.

The influence of initial bacterial counts and heat treatment of raw whole milk and the initial pH of sterile 10% NDM on growth of S. aureus and production of enterotoxin A was evaluated by Tatini et al., (1971). They reported that, in milk relatively free from competing organisms, an S. aureus population of $2x10^6$ to $3x10^6$/ml was associated with detectable enterotoxin A. Toxin was detected after 4 to 6 hr in raw milk with low bacterial counts and in sterile milk, inoculated initially with 10^3 to 10^5 S. aureus cells/ml. Enterotoxin A was produced in sterile milk at initial pH values of 4.5 to 6.5 (adjusted with hydrochloric acid). At pH 4.5, lactic acid was bactericidal to S. aureus.

Factors Affecting *Staphylococcus aureus* Growth and Enterotoxin Production

1. Effect of moisture

Staphylococci can grow at low water activity (a_w) or high osmotic pressure. Segalove and Dack (1951) reported growth of enterotoxigenic *S. aureus* strain 161 in dehydrated beef and pork at 30% moisture. Scott (1953) studied growth of *S. aureus* at 30 C with different water activities. He observed growth of *S. aureus* at 0.86 a_w but reported no growth below 0.86 a_w, at 30 C, during 30 days of incubation.

2. Effect of oxygen availability

Staphylococci are facultative, however, growth and enterotoxin production have been reported to be better under aerobic conditions (Kato et al., 1966). Thatcher, Robinson, and Erdman (1962) reported significant production of enterotoxin A under reduced oxygen tension and in vacuum packed bacon.

3. Effect of temperature

The temperature range for enterotoxin production by *S. aureus* 161, was 4 to 37 C (Segalove and Dack, 1941). McLean, Lilly and Alford (1968), working with *S. aureus* 243 observed a reduction in amounts of enterotoxin B as the incubation temperature decreased below 37 C. Production of enterotoxin A by *S. aureus* MF 224, also decreased at lower incubation temperatures (Donnelly et aL, 1968).

4. Effect of certain minerals and nutrients

Staphylococcus aureus grew and produced enterotoxin upon inoculation in a medium containing glucose, ammonium sulfate, and other minerals, nicotinic acid, thiamine, and only two or three amino-acids (Peters, 1964). In the absence of glucose in minimal growth medium, enterotoxin B was produced after 72 hr. Although glucose increased the rate of production of enterotoxin B, it was not essential.

Kliger, Grossowicz, and Bergner (1943) reported that nicotinic acid and thiamine were essential for staphylococcal growth. Biotin was essential only when S. aureus S_6 was grown on medium containing glutamic acid as the sole carbon source in place of glucose (Mah, Fung, and Morse, 1967).

Freidman (1966, 1968) observed that Mg^{++} was required for the enterotoxin B production by S. aureus S_6. He indicated that K^+ and sodium fluoride inhibition was reversed by Mg^{++}; and postulated that inhibition of S. aureus by K^+ might be due to interferrence in Mg^{++} uptake, while that caused by sodium fluoride was due to the formation of an irreversible complex with Mg^{++} requiring enzymes.

Markus and Silverman (1970) noted that toxin production per unit growth was not affected by sodium nitrite, sodium nitrate, or sodium chloride. Toxin secretion was also increased by several surfactants but the secretion by non-replicating cells was inhibited by chloramphenicol and 2,4, dinitrophenol, and was not affected by streptomycin or penicillin G.

5. Effect of pH of growth medium

Staphylococci grow over a pH range of 4.0 to 10.0 (Indolo, Ordal, and Witter, 1964). Casman et al., (1967) grew S. aureus at pH 7.0 for production of enterotoxin D and in an earlier report (Casman and Bennett, 1963) observed that enterotoxin A could be produced at pH 5.3 to 5.5. Reisser and Weiss (1969) determined the effect of initial pH and length of incubation time at 37 C, in four different growth media, on the production of staphylococcal enterotoxins A, B and C. After 24 hr an initial pH of 6.8 gave higher yields of enterotoxin B and C than pH 6.0 or 5.3, and lower initial pH values did not affect production of enterotoxin A. Prolonged incubation (48 to 72 hr) resulted occasionally in higher yields of enterotoxins. Their results indicated that enterotoxin production was always associated with an increase in pH. All strains raised the pH of media, from 5.3 during 24, 48 or 72 hr incubation period, except S. aureus 137, an enterotoxin C producing strain.

Markus and Silverman (1969) employed non-replicating cells of S. aureus in their study. This procedure separated the requirements of enterotoxin B secretion from those of growth and replication. They reported that optimum pH for secretion of enterotoxin A by non-replicating cells was 6.5 to 6.8.

Genigeorgis et al., (1971a) attempted to study the probability of initiating aerobic growth of staphylococci at 30 C in broths with different initial pH and salt

concentrations. They designed the experiment in factorial fashion involving four strains of staphylococci producing enterotoxins A, B, C, and D, five salt concentrations and six initial pHs. They developed a regression equation for each strain of staphylococci which enabled them to predict the population at any combination of pH and salt concentration. These regression equations appeared to be medium dependent as those derived by using Brain Heart Infusion broth as medium could not be used correctly for predicting the staphylococcal population in meats (Genigeorgis, Savoukidis and Martin, 1971b).

Minore and Marth (1970) reported the effect of gradually reducing the pH of pasteurized milk with acetic, citric, hydrochloric, lactic, and phosphoric acids, over a period of 4, 8, and 12 hr on the growth of S. aureus 100. In addition, the effects of 1:1 mixtures of lactic and each of the other acids, and of acetic and citric acid were evaluated using this organism. A 99% reduction in growth over 12 hr period was obtained with a final pH value of 5.0 for acetic, 4.6 for lactic, 4.5 for citric, 4.1 for phosphoric and 4.0 for hydrochloric acid. The mixture of acids adjusted to pH values at the border line for growth exhibited neither synergistic nor antagonistic effects between two acids.

6. Effect of presence of micro-organisms

Oberhofer and Frazier (1961) studied the effect of simultaneous growth of other competing micro-organisms on

two enterotoxigenic and two nonenterotoxigenic strains of S. aureus in milk free media. They reported screening of 66 food microorganisms by spot-tests on their ability to influence the growth of S. aureus. The most consistantly inhibitory cultures for S. aureus were, Streptococcus faecium, S. faecalis, S. faecalis var liquifaciens a nisin producing strain of S. lactis and various meat lactobacilli. Graves and Frazier (1963) isolated 870 cultures of predominating micro-organisms from various types of foods and reported the growth abilities of S. aureus 196E by spot-tests. They observed that most of the lactic acid bacteria were inhibitory.

Type of medium and temperature of incubation were important environmental factors for growth inhibition of S. aureus by food bacteria. The repression of S. aureus growth was maximum in pH range of 6.4 to 7.2; and the ratio of inhibitor bacteria to staphylococci was an important factor in growth inhibition (Troller and Frazier, 1963).

The effect of coliform and Proteus bacteria on growth of S. aureus 196E was studied by DiGiancinto and Frazier (1966). They observed that staphylococci did not reach $5x10^6$ cells/ml in trypticase soy broth at 10, 15, 22 or 30 C when the ratio of inhibitor bacteria to staphylococci was 100:1. They assumed that $5x10^6$ cells of S. aureus/ml was the minimum number necessary to cause food poisoning. They reported that the greatest overall inhibition was

produced by Escherichia coli, E. freundii and Proteus vulgaris. These species were more inhibitory than others both at 22 and 30 C.

Indolo et al., (1965) reported repression of S. aureus MF 31, by Streptococcus diacetylactis and other lactic streptococci. Their data indicated that the initial proportion of staphylococci present in the medium was of less importance than depletion of vital nutrients. They indicated that factors present in yeast nitrogen base medium could reverse the inhibition. The major factor was found to be nicotinamide and their study indicated that biological availability of this compound was pH dependent. The addition of nicotinamide to depleted media with careful pH control, increased the S. aureus growth.

The growth of S. aureus in presence of lactic streptococci was reported by Kao and Frazier (1966). Staphylococcus aureus 196E, an enterotoxigenic isolate, and strain W-1 a non-enterotoxigenic strain isolated from a mastitic udder were used. Kao and Frazier observed that the cultures of lactic acid bacteria, mostly isolated from food, affected the growth of S. aureus in trypticase soy broth. Some of these organisms (Streptococcus faecalis, S. faecium, Lactobacillus lactis, and Leuconostoc mesenteroides,) stimulated the growth of S. aureus during the early hours, especially at higher incubation temperature. However, most cultures were inhibitory and some (S. faecium and L. mesenteroides) were bactericidal upon attainment of their maximal growth. They also

observed that meat lactobacilli and Leuconostoc dextranicum inhibited S. aureus at 10, 15, 20 and 25 C. Their results indicated that the effect of these bacteria on both S. aureus strains was similar.

7. Effect of nisin and hydrogen peroxide

Nisin, an antibiotic, produced by strains of S. lactis was reported to be inhibitory to certain staphylococci, bacilli, and clostridia (Mattick and Hirsch, 1947). Jones (1970, unpublished) observed inhibition of S. aureus strains capable of producing enterotoxins A, B, C, and D by S. lactis 222, a nisin producing strain.

Hydrogen peroxide was observed to inhibit the growth of staphylococci when grown in association with lactobacilli (Dahiya and Speck, 1968). Dahiya and Speck used calcium carbonate fortified trypticase soy agar to neutralize inhibition due to acid. They observed that S. aureus MF 31, was completely inhibited by 6 µg of hydrogen peroxide per milliliter. They noted that the high accumulation of hydrogen peroxide in statically incubated flask, during early hours of acid production, completely inhibited S. aureus MF 31. Amin and Olson (1967) observed that in milk upon treatment with 500 µg of hydrogen peroxide per milliliter at 37·8 C, S. aureus 196E was completely inhibited. Later (1968) they reported that S. aureus 196E had a higher catalase activity than other strains. This observation provided an explanation for variable inhibitory response of staphylococci to hydrogen peroxide concentrations.

Speck and Gilliland (1971) noted that lactic streptococci do not produce sufficient quantities of hydrogen peroxide to be inhibitory to staphylococci.

MATERIALS AND METHODS

1. Staphylococcus aureus strains 13665, 14485, 19095, and 23235, capable of producing enterotoxins A, B, C, and D, respectively, were obtained from the American Type Culture Collection. These strains were maintained in Brain Heart Infusion (BHI) agar (Difco) slants, and transferred every two months by incubating at 37 C for 24 hr and then holding at 5 C.

2. Streptococcus lactis strains AM_2 and ML_8 were obtained from Dr. C. A. Ernstrom, Department of Food Science and Industries, who obtained them from the New Zealand Dairy Research Institute. Strain 222 was obtained from Dr. A. Hurst Research Laboratories, Food and Drug Directorate, Ottawa, Canada. Strains AM_2 and ML_8 were subcultured every week in 10%, sterile, non-fat dry milk (NDM) at 21 C for 16-18 hr and stored at 5 C. Strain 222 was transferred weekly, in Elliker broth, by incubating at room temperature for 24 hr and storing at 5 C.

3. A limited supply of enterotoxin-D and antienterotoxin-D was the kind gift of Dr. J. C. Olson, Jr., of the F.D.A., Washington, D.C. Enterotoxin-D was also prepared as described by Casman et al., (1967) except that after obtaining a 10% pure preparation removal of hemolysins was not carried out. Hemolysins were inactivated by incubating the preparation with 0.6% formaldehyde at 37 C for one week

(Bergdoll, 1966). Antienterotoxin-D was prepared by following the immunization schedule presented in Table 3. One week after the last injection the animals were bled by cardiac-puncture. The blood was allowed to coagulate for 2 hr at room temperature and the serum was obtained by centrifuging at 1,000 g for 30 min (Campbell et al., 1964). Antibody titer was found to be 1:80, by precipitation test when antigen concentration was 1:500 (Carpenter, 1956). Serum was lyophilized after addition of Merthiolate to obtain a final concentration of 1:10,000 and was stored at -4 C (Campbell et al., 1964).

4. During cheese preparation, the yield is approximately 10%. Thus concentration of buffering constituents in 10 g of cheese is approximately equal to that of 100 ml of milk. Due to this reason, therefore, 100 ml of milk were mixed with 50 ml of 0.2M sodium chloride (pH 7.4) and enterotoxin purification from milk samples were carried out as described by Zehren and Zehren (1968a). Enterotoxin D was assayed by the method of Gandhi and Richardson (1971).

5. Non-fat dry milk powder was reconstituted in distilled water to 10% milk solids and autoclaved at 15 lb steam pressure for 12 min. The medium was first inoculated with 24 hr old cultures of S. aureus, followed by different percentage of lactic starter. The lactic starter was a 2:1 blend of S. lactis strains AM_2:ML_8, previously cultured separately in sterile 10% sterile NDM at 21 C for 16-18 hr. The flasks were incubated at 32 C for 24 hr. The pH of this

Table 3. Immunization schedule for production of antienterotoxin-D in rabbits

Date	Micrograms of enterotoxin-D injected[a]	Route	Comments
30 July 1970	1.0	Foot-pad	With aluminum phosphate adjuvant[b]
6 Aug. 1970	2.0	Food-pad	With aluminum phosphate adjuvant
13 Aug. 1970	4.0	Foot-pad	With aluminum phosphate adjuvant
20 Aug. 1970	10.0	Foot-pad	With aluminum phosphate adjuvant
27 Aug. 1970	20.0	Foot-pad	With aluminum phosphate adjuvant
		Two-week rest period	
11 Sept.1970	100.0	Intra-peritonial (I.P.)	With aluminum phosphate adjuvant
17 Sept.1970	100.0	Intra-peritonial (I.P.)	With aluminum phosphate adjuvant
22 Sept.1970	200.0	Intra-peritonial (I.P.)	With aluminum phosphate adjuvant
25 Sept.1970	400.0	Intra-peritonial (I.P.)	Without adjuvant
28 Sept.1970	---	-----	Titer 1:20
14 Oct. 1970	500.0	Intra-peritonial (I.P.)	Without adjuvant
21 Oct. 1970	---	-----	Titer 1:80

[a]Antigen was formalized at 37 C for one week by treating 10% pure preparation with 0.6% formaldehyde in order to inactive hemolysins (Bergdoll, 1966).

[b]The formalized antigen was mixed with adjuvant following the procedure of Gorden et al., (1957).

mixture was determined at 2 hr intervals from 0 to 8 hr and at 24 hr. A 10 ml sample of this mixture was transferred into a disposable cup and the pH was determined with Corning, Model-7 pH meter.

The staphylococcal counts were determined from milk by adopting a microtiter technique (Fung and Kraft, 1969) in which serial dilutions were carried out rapidly in microtiter plastic plates (Cooke Engineering Co., Alexandria, Va.). Sterile, quarter strength Ringer's solution was used for milk sample dilution as described by Harrigan and McLane (1966). The enumeration of staphylococci was carried out on S-110 (Difco) medium. Golden-yellow colonies on S-110 medium, after incubation at 37 C, for 48 hr, and then at room temperature for 24 hr, were reported as S. aureus.

The data obtained for pH and staphylococcal population in logarithm to base ten were subjected to regression analysis as described by Snedecor (1956).

7. Nisin producing ability of S. lactis strains, AM_2 and ML_8 were studied by a modification of the method described by Tanaka et al., (1967). They allowed the streptococci to grow for 2 hr at 32 C and then the plates were overlaid with a Bacillus stearothermophilus strain and incubated at 55 C. In this study, broth was used in place of agar and the strain of test organism B. stearothermophilus 1596 was adopted. Results were compared with a known nisin producing strain of S. lactis (222) and absorbancy (A) was measured at 600 nm.

8. The inhibitory action of S. lactis strains AM_2, ML_8 and 2:1 blend (V/V) of AM_2:ML_8 was studied by modifying a method of Dahiya and Speck (1968). Trypticase soy agar (1.5%) of Difco, was allowed to solidify in petri plates. Five milliliters of trypticase soy agar (0.5%) containing 0.1 ml of 24 hr old S. aureus strains, and 1 ml of sterile 20% calcium carbonate was overlaid after thorough mixing. After solidification of the overlayer agar, 0.03 ml of S. lactis strains AM_2, ML_8, 222 and 2:1 blend (V/V) of AM_2:ML_8 were spotted. Streptococcus lactis strains AM_2, ML_8 and 222 were grown separately, at 32 C for 8 hr in Elliker broth prior to spotting. The plates were incubated at 32 C for 24 hr after spotting. The degree of inhibition of S. aureus strains was determined by the absence of growth around the spots.

Inhibitory response on trypticase soy agar and on nutrient agar without calcium carbonate fortification was also carried out as a check for inhibition due to acid.

9. Hydrogen peroxide production by S. lactis strains AM_2, ML_8 and 222 was evaluated by using Peroxystrips (Miles Chemical Company, Elkhart, Indiana). The strains AM_2, ML_8 and 222 were inoculated into 10 ml Elliker broth and were incubated at 32 C separately. At 4, 6 and 8 hr, a 1 ml quantity of culture was transferred into sterile tubes and was tested for hydrogen peroxide production by dipping Peroxystrips into the medium. The production of H_2O_2 was

observed by a blue color development on the strips, indicating a positive test and thereby showing the presence of 5 µg or greater concentration of hydrogen peroxide/ml. The Peroxystrips were tested in 5 µg/ml of hydrogen peroxide solution to assure that no loss in sensitivity had occurred.

RESULTS AND DISCUSSION

Influence of Acid Production Rate by Starter on S. aureus Growth

The effect of reduced starter levels on approximately 10^6 cells of S. aureus 23235/ml, an enterotoxin D producing strain, in sterile, 10% NDM at 32 C during 24 hr, is presented in Table 4 and Figure 1. The pH of the starter free milk did not vary much during the 0 to 24 hr incubation period. It nearly remained constant for all starter levels during 0 to 4 hr incubation except for 1% and 0.5% starter levels where it dropped slightly. The pH drop, beyond 4 hr, was evident for all starter levels used; and was proportional to the starter inocula.

The 1% and 0.5% starter levels reduced the pH of milk very rapidly from 4 to 8 hr; while a slight decrease in pH was observed beyond 8 hr. The pH decreased from 4 to 8 hr for 0.1% starter level, with a sharp decrease from 6 to 8 hr; and the pH drop beyond 8 hr, was gradual. Depending upon the starter concentrations, the pH dropped slightly for 0.01% and for 0.001% starter levels during 4 to 6 hr period and decreased gradually during 6 to 8 hr.

The staphylococcal population, remained nearly constant from 0 to 4 hr, with an increase in growth at 6 hr, which was observed for all starter levels except for 1% and 0.5% starter levels which did not allow the increase. From 6 to 8

Table 4. Effect of reduced starter levels on high inoculum of S. aureus 23235 in sterile milk (10% NDM) during 24 hr at 32 C

Exp.	Starter %	[a] S. aureus 23235 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.	pH 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1	1.0	4.964	4.768	4.682	4.947	4.688	3.288	6.45	6.42	6.22	5.45	4.80	4.15
	0.5	5.100	4.642	4.682	4.479	5.014	3.398	6.45	6.45	6.30	5.75	4.95	4.15
	0.1	4.940	-[b]	-	5.143	5.699	4.300	6.42	6.42	6.38	6.12	5.35	4.20
	0.01	5.042	4.916	-	5.882	5.699	8.699	6.45	6.48	6.45	6.40	5.90	4.20
	0.001	5.048	4.796	-	5.917	5.699	8.965	6.45	6.48	6.45	6.40	6.05	4.20
	0.000	5.061	4.615	5.000	5.976	6.699	10.014	6.45	6.48	6.48	6.50	6.50	6.25
2	1.0	6.578	6.759	6.558	6.869	6.588	3.954	6.35	6.15	5.90	5.22	4.55	4.20
	0.5	6.513	6.558	6.699	6.677	6.822	3.982	6.30	6.15	6.00	5.35	4.65	4.18
	0.1	6.300	6.653	6.374	7.072	7.114	4.678	6.35	6.28	6.25	5.90	5.15	4.20
	0.01	6.000	6.438	5.995	6.759	7.740	8.038	6.35	6.28	6.25	6.15	5.70	4.20
	0.001	6.237	6.496	6.699	7.079	8.316	9.300	6.35	6.28	6.28	6.25	6.15	4.20
	0.000	6.439	6.618	6.875	8.000	8.699	10.699	6.38	6.28	6.28	6.30	6.30	6.18

[a] S. aureus numbers are in log values.

[b] No observation taken

$$\Delta staph = 0 = 3.5998 - 4.2125\ \Delta pH$$

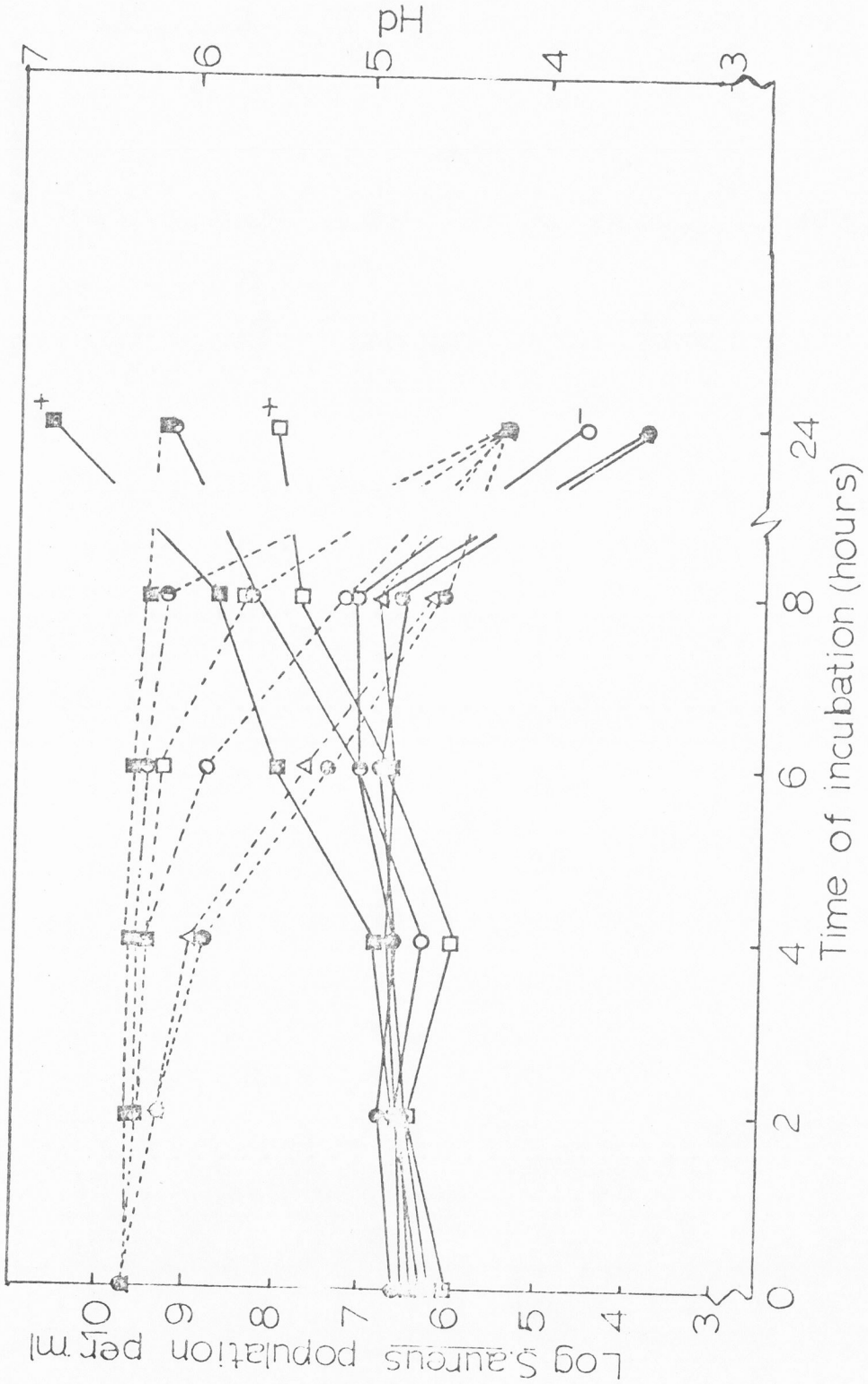
$$\Delta pH = \frac{3.5998}{4.2125} = 0.852$$

$$\hat{y} = \Delta staph\ (24 - 6) = b_o + b_i x$$

$$x = \Delta pH\ (4 - 8)$$

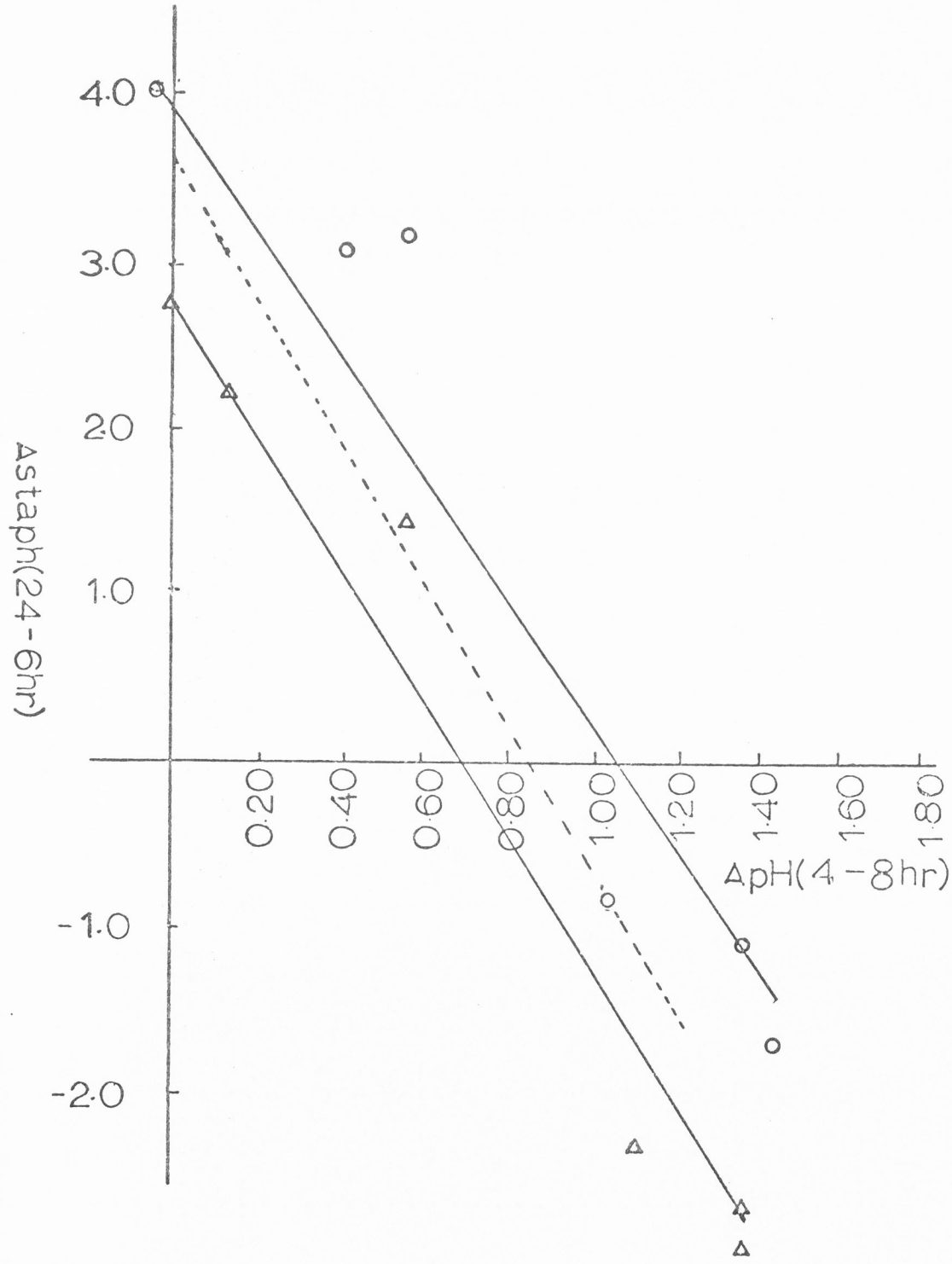
$$= 3.5998 - 4.2125\ \Delta pH$$

γ = correlation coefficient = -0.93


pH
7
6
5
4
3
+
+
-
Log S.aureus population per ml
0
9
8
7
6
5
4
3
0
2
4
6
8
24
Time of incubation (hours)

hr the staphylococcal population increased where starter levels were lower than 1% and 0.5%. A starter level of 0.1% allowed a slight increase in S. aureus population during 6 to 8 hr; while 0.01%, and 0.001% starter levels allowed a sharp increase in S. aureus population. At 24 hr the staphylococcal population decreased in presence of 1%, 0.5% and 0.1% starter levels; and increased in presence of 0.01, and 0.001% starter levels. The increase of S. aureus population was gradual beyond 8 hr, but the rate of increase was lower as starter concentration increased from 0.001 to 0.01%.

The increase in staphylococcal population beyond 6 hr was inversely related to the drop in pH from 4 to 8 hr (Correlation coefficient = γ = -0.93). The drop in pH (Figure 2) from 4 to 8 hr (ΔpH) was linearly related to the logarithmic change in staphylococcal population from 6 to 24 hr (Δ staph = S. aureus population at 24 hr - that at 6 hr). The dotted line, representing the regression line, intercepts at the ΔpH value of 0.85. This indicated that during 8 hr incubation, if pH dropped by 0.85 units from that at 4 hr then there would be no change in S. aureus population. Thus, this pH value would be in the range of 5.4 to 5.7, if the pH at 4 hr was 6.25 to 6.55 respectively. The regression line (Figure 2) and the regression equation (Table 4) could be useful in predicting the S. aureus population change from 6 to 24 hr. Thus if, ΔpH was 0.30, then a predicted Δ staph would be 2.45, indicating an


4.0
3.0
2.0
1.0
-1.0
-2.0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
ΔpH(4-8hr)
Δstaph(24-6hr)

increase in S. aureus population by 2.45 log from 6 hr to 24 hr. Similarly if ΔpH was 1.10 then, Δ staph would be -1.3, indicating a decrease in S. aureus population by 1.3 log from 6 hr to 24 hr. The regression equations reported subsequently could also be useful in predicting the change in S. aureus population from 6 hr to 24 hr under the conditions used in this study.

A preliminary study in pasteurized milk indicated that, at ΔpH of 0.30 to 0.40, change in staphylococcal population from 6 to 24 hr would be zero under the similar conditions. Further studies were necessary to confirm these results as uninoculated pasteurized milk control also reduced pH to 4.30 from 6.80, after 24 hr incubation period at 32 C, which could be attributed post-pasteurization contamination of lactic streptococci.

The 24 hr samples with an increase in viable counts were subjected to enterotoxin analysis. The positive sign (+) (Figure 1) indicates 10 or more μg of enterotoxin D per 100 ml of milk sample. Enterotoxin D was not detected in samples where 0.1% starter was used [Negative sign (-)] and was not analyzed in the other samples (No sign). In samples containing 0.01% starter inocula, staphylococci did not grow beyond 8 hr as rapidly as they grew during 4 to 8 hr period and still produced enterotoxin D. Thus, the drop in pH from 4 to 8 hr could be an important factor, not only in

determining the population change of S. aureus during 6 to 24 hrs, but, most likely, also in preventing formation of enterotoxin D.

The effect of two starter inocula, one known to decrease S. aureus population effectively at 24 hr, and the other known to allow growth of S. aureus (0.1% and 0.01% respectively) are referred to as critical starter inocula (Table 4 and Figure 1).

The effect of these critical starter inocula upon the reduced levels of S. aureus 23235 is presented in Table 5 and Figure 3. Approximately 10^6 cells of S. aureus 23235/ml were decreased to about 10^4 cells/ml, at 24 hr, by 0.1% starter inocula; while approximately 10^4 cells/ml neither decreased nor increased. A starter level of 0.01% allowed increase in both cases. Approximately 10^2 cells of S. aureus/ml neither increased nor decreased in the presence of 0.01% starter inoculum. The regression equation (Table 5) indicated a ΔpH value of 0.82.

The effect of critical starter concentrations on S. aureus strains capable of producing enterotoxins A, B, C and D is presented in Table 6. The population of S. aureus at 24 hr indicated differential behavior of all four strains. Even 0.01% starter inoculum decreased populations of enterotoxin A and C producing strains of S. aureus and allowed a slight increase in population of enterotoxin B producing

Table 5. Effect of critical starter inocula on reduced levels of S. aureus 23235 in sterile milk during 24 hr at 32 C

Exp.	Staph	Starter %	[a] S. aureus 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.	pH 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1	High	0.1	5.874	5.874	6.399	7.030	7.432	4.944	6.45	6.40	6.35	6.00	5.35	4.20
		0.01	5.983	6.090	6.510	6.708	7.254	7.615	6.45	6.42	6.40	6.30	5.90	4.22
		0.00	5.708	5.788	6.090	7.741	-[b]	9.399	6.50	6.45	6.42	6.40	6.40	6.35
	Medium	0.1	2.940	3.498	3.826	4.122	4.734	3.806	6.42	6.42	6.42	6.15	5.42	4.20
		0.01	3.078	3.478	4.036	4.128	4.734	5.000	6.45	6.42	6.42	6.35	6.05	4.20
		0.00	3.302	3.432	4.022	4.793	5.638	9.194	6.48	6.45	6.45	6.42	6.42	6.32
	Low	0.1	1.000	1.000	-	1.699	1.958	1.768	6.45	6.40	6.38	6.12	5.42	4.20
		0.01	1.000	1.000	2.000	1.944	2.794	2.343	6.45	6.45	6.40	6.35	6.05	4.20
		0.00	1.000	1.000	1.778	2.778	3.564	9.578	6.45	6.42	6.42	6.40	6.40	6.30
2	High	0.1	6.146	6.564	6.190	6.432	7.218	4.699	6.42	6.35	6.30	5.90	5.15	4.20
		0.01	6.699	6.653	6.108	6.182	6.338	8.155	6.42	6.35	6.35	6.20	5.75	4.25
		0.00	6.343	6.859	7.134	7.453	7.652	9.344	6.42	6.35	6.35	6.38	6.35	6.35
	Medium	0.1	4.194	4.496	4.790	4.764	4.786	4.443	6.45	6.35	6.30	5.95	5.20	4.20
		0.01	4.228	4.528	4.872	5.018	5.548	6.477	6.45	6.35	6.35	6.22	5.80	4.20
		0.00	4.343	4.615	5.004	5.176	6.246	9.224	6.45	6.35	6.35	6.35	6.35	6.32
	Low	0.1	2.278	2.146	2.673	2.278	2.699	-	6.45	6.38	6.32	6.00	5.30	4.20
		0.01	2.078	2.545	2.742	2.832	3.010	2.078	6.45	6.38	6.35	6.25	5.95	4.20
		0.00	2.078	2.492	2.634	3.453	4.324	9.366	6.45	6.38	6.38	6.38	6.35	6.32

[a] S. aureus numbers are in log values.

[b] No observation taken.

$\Delta staph = \hat{y} = 3.1656 - 3.839\ \Delta pH$

$= 0 = 3.1656 - 3.839\ \Delta pH$

γ = Correlation Coefficient = -0.615

$\Delta pH = 0.821$

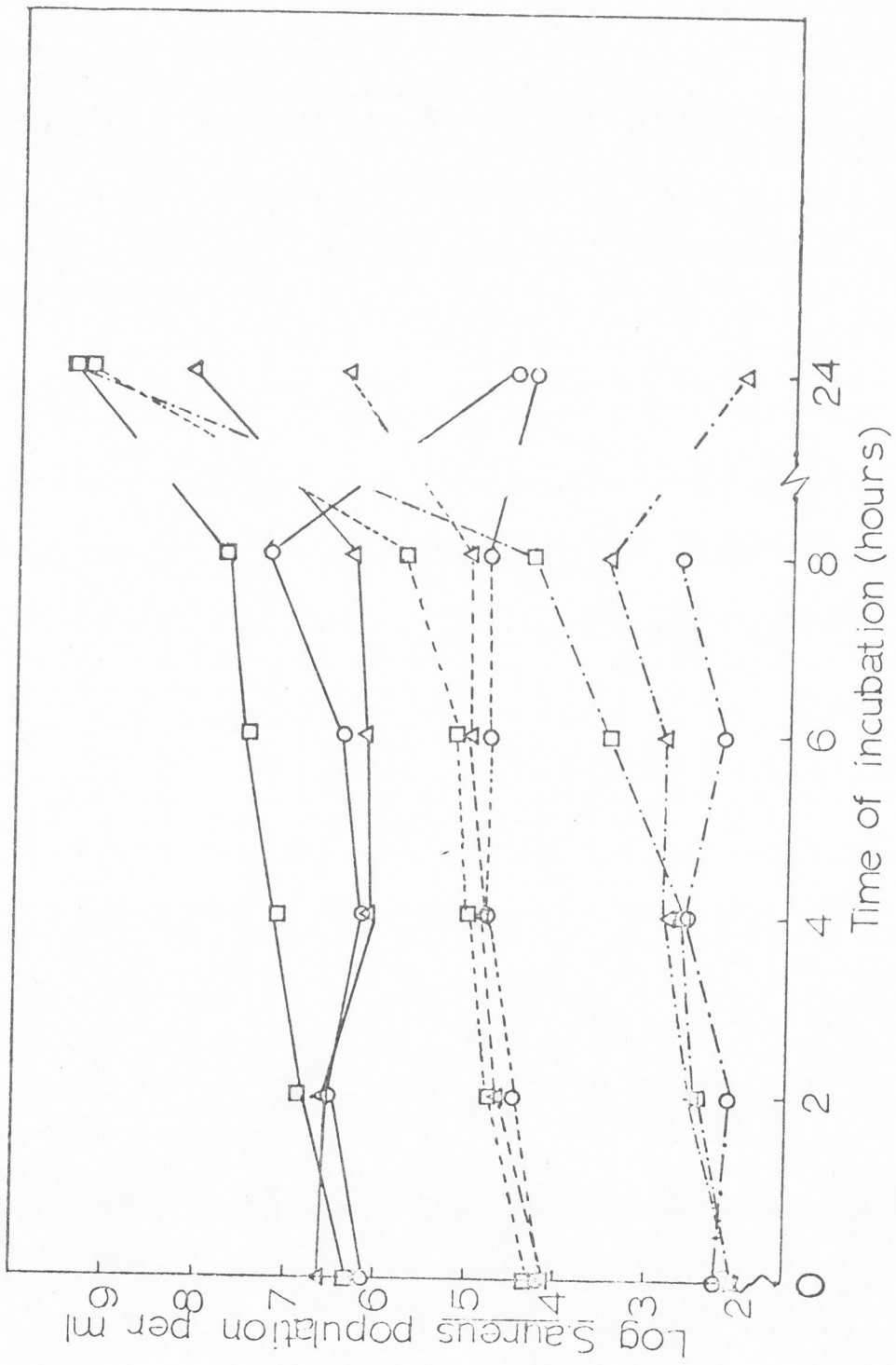

Log S.aureus population per ml
2
3
4
5
6
7
8
9
0
2
4
6
8
24
Time of incubation (hours)

Table 6. Effect of critical starter inocula on high levels of S. aureus 13665, 14458, 19095, and 23235 in sterile milk during 24 hr at 32 C

Exp.	Staph	Starter %	[a] S. aureus 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.	pH 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
		0.1	5.677	5.628	5.422	6.999	-[b]	-	6.50	6.48	6.40	5.90	5.20	4.30
	A	0.01	5.575	5.740	5.398	-	5.175	-	6.52	6.48	6.45	6.30	5.75	4.28
		0.00	5.602	5.439	6.000	-	-	8.352	6.52	6.50	6.48	6.45	6.48	6.42
		0.1	6.168	5.829	6.699	6.105	5.875	-	6.52	6.45	6.35	5.90	5.15	4.30
	B	0.01	6.061	5.572	6.114	6.813	7.628	-	6.50	6.45	6.45	6.25	5.65	4.30
1		0.00	5.398	5.699	6.032	6.889	8.447	10.504	6.50	6.48	6.48	6.48	6.45	6.30
		0.1	5.903	5.740	6.152	6.196	6.114	4.225	6.48	6.42	6.35	5.95	5.22	4.20
	C	0.01	5.968	5.542	6.162	5.778	6.190	-	6.50	6.48	6.45	6.25	5.72	4.30
		0.00	6.114	5.796	6.190	6.197	6.190	8.720	6.50	6.45	6.45	6.45	6.45	6.35
		0.1	5.875	5.875	6.398	7.030	7.231	4.944	6.45	6.40	6.35	6.00	5.35	4.20
	D	0.01	5.983	6.090	6.510	6.709	7.255	7.625	6.45	6.45	6.42	6.35	6.05	4.20
		0.00	5.708	5.778	6.098	7.741	-	9.398	6.45	6.48	6.48	6.50	6.50	6.25
		0.1	5.198	5.172	6.069	6.741	6.829	-	6.55	6.50	6.35	5.85	4.85	4.30
	A	0.01	5.039	5.882	6.021	6.439	7.236	2.725	6.55	6.50	6.45	6.28	5.45	4.30
		0.00	5.060	5.122	6.478	7.778	7.575	10.498	6.55	6.55	6.50	6.45	6.45	6.25
		0.1	5.512	5.903	6.031	6.386	6.889	4.776	6.55	6.50	6.35	5.85	4.95	4.28
	B	0.01	5.223	5.942	6.872	7.439	7.720	5.775	6.55	6.52	6.45	6.25	5.50	4.28
2		0.00	5.439	6.114	6.778	7.553	8.336	10.470	6.55	6.55	6.55	6.52	6.52	6.30
		0.1	5.138	5.455	6.439	6.224	6.889	3.080	6.55	6.58	6.38	5.85	4.90	4.28
	C	0.01	5.224	5.511	6.954	7.032	7.182	4.458	6.55	6.55	6.50	6.40	5.55	4.28
		0.00	5.476	5.573	7.321	7.069	8.338	9.185	6.55	6.55	6.52	6.52	6.48	6.38
		0.1	5.860	5.778	6.798	7.021	7.176	4.732	6.58	6.55	6.45	5.85	4.95	4.30
	D	0.01	5.332	5.478	6.625	7.196	7.439	7.916	6.55	6.52	6.48	6.35	5.58	4.30
		0.00	5.740	5.720	6.674	8.070	8.332	10.544	6.58	6.55	6.55	6.55	6.50	6.40

[a] S. aureus numbers are in log values.

[b] No observation taken.

γ = Correlation Coefficient = -0.78

$\hat{y}$ = Δstaph (24 - 6) = 2.8761 $\pm$ 5.3897 x ΔpH (4 - 8)

if Δstaph = 0
then ΔpH = 0.535

strain at 24 hr. This indicated a differential susceptibility of all four strains to the metabolites excreted by starter in sterile milk.

The data obtained for approximately 10^6 cells of S. aureus 23235/ml, reported in Tables 4, 5 and 6, are presented in Table 7. The regression analysis indicated same ΔpH value of 0.82 reported earlier for this organism (Table 5).

The rate of acid production and starter activity were important in preventing staphylococcal growth and thereby enterotoxin production. The results of the present investigation were in agreement with findings of Jezeski et al., (1967), Reiter et al., (1964) and Zehren and Zehren (1968b). The lactic phage attack allowed S. aureus growth and thereby enterotoxin production (Jezeski et al., 1967).

In the present study, the severity of lactic phage attack was predicted by using reduced starter inocula. A mild phage attack resulting in survival of only 10%, or 1.0% active lactic culture was assumed. Thus, when only 10% active lactic culture was viable, or when 0.1% of starter inoculum was used; staphylococci did not increase in sterile 10% NDM, at 32 C during 24 hr. Some S. aureus strains did not increase even in 1% active viable culture, or 0.01% starter inoculum, in sterile milk.

Thus, if 1% lactic inoculum is used in sterile milk, and due to a very mild phage attack if only 90% of the

Table 7. Compilation of data for high levels of S. aureus 23235, given in Tables 4, 5 and 6 for critical starter inocula

Exp.	Starter %	[a] S. aureus 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.	pH 0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.
1	0.1	4.904	-[b]	-	5.144	5.699	4.302	6.42	6.42	6.38	6.12	5.35	4.20
	0.01	5.041	4.916	-	5.882	5.699	8.699	6.45	6.48	6.45	6.40	5.90	4.20
	0.00	5.071	4.614	5.000	5.976	6.699	10.140	6.45	6.48	6.48	6.50	6.50	6.25
2	0.1	6.302	6.654	6.376	7.074	7.114	4.668	6.35	6.28	6.25	5.90	5.15	4.20
	0.01	6.000	6.439	5.989	6.160	7.740	8.037	6.35	6.28	6.25	6.15	5.70	4.20
	0.00	6.423	6.618	6.875	8.000	8.699	10.699	6.35	6.28	6.30	6.30	6.30	6.18
3	0.1	5.875	5.875	6.398	7.030	7.231	4.944	6.45	6.40	6.35	6.00	5.35	4.20
	0.01	5.983	6.090	6.570	6.709	7.255	7.615	6.45	6.45	6.42	6.35	6.05	4.20
	0.00	5.071	4.614	5.000	5.976	6.699	10.140	6.45	6.48	6.48	6.50	6.50	6.25
4	0.1	6.146	6.574	6.191	6.432	7.218	4.699	6.42	6.35	6.30	5.90	5.15	4.20
	0.01	6.990	6.653	6.100	6.182	6.348	8.155	6.42	6.35	6.35	6.22	5.80	4.22
	0.00	6.342	6.858	7.132	7.452	7.650	9.342	6.45	6.35	6.35	6.38	6.35	6.32
5	0.1	5.860	5.778	6.798	7.021	7.176	4.732	6.58	6.55	6.45	5.85	4.95	4.30
	0.01	5.332	5.478	6.625	7.196	7.439	7.916	6.55	6.52	6.48	6.35	5.58	4.30
	0.00	5.740	5.720	6.574	8.070	8.332	10.544	6.58	6.55	6.55	6.50	6.50	6.40

[a] S. aureus numbers are in log values.

[b] No observation taken.

$\Delta staph = \hat{y} = 3.3041 + 4.045\ \Delta pH\ (4 - 8)\ (24 - 6)$

If $\Delta staph = 0$ then $\Delta pH = 0.821$

γ = Correlation Coefficient = -0.895

starter is destroyed; then the remaining 10% of active starter culture would most likely prevent any increase in S. aureus population.

Evaluation of Nisin Production

When pH of sterile, 10% NDM, was adjusted to 5.0 or higher, by lactic acid alone; S. aureus growth was observed at 30 C (Reiter et al., 1964; Tatini et al., 1971). The present studies, by using a 2:1 blend of AM_2:ML_8 strains of S. lactis, showed that at pH 5.40 (if, ΔpH = pH at 4 hr - that at 8 hr = 0.82 and, if pH at 4 hr was 6.22), S. aureus population did not change during 6 to 24 hr incubation period. Thus it is evident that lactic organisms produce some inhibitor other than lactic acid alone. This observation was in agreement with that of Reiter et al., (1964).

Nisin, an antibiotic produced by lactic streptococci, has been known to inhibit S. aureus growth (Mattick and Hirsch, 1947). Nisin production by S. lactis strains AM_2, ML_8, and a 2:1 (N/N) blend of AM_2:ML_8 was studied (Table 8). The results were compared with a known nisin producing S. lactis strain (222). The control column indicates the absorbancy at 600 nm for the lactic cultures following growth for 2 hr at 32 C. Nisin production was evident by decrease in absorbancy. Streptococcus lactis 222 produced nisin, and the production rate increased as the quantity of lactic inocula, used in assay medium, was increased.

Table 8. Evaluation of nisin production by S. lactis AM_2, ML_8, 222, and 2:1 (V/V) blend of AM_2:ML_8 strains in nutrient broth using B. stearothermophilus at 55 C for 24 hr

S. lactis strains	ml. of lactic culture	B. st.[a]	Control	Difference[b]
AM_2	0.01	0.190[c]	0.005	0.185
	0.02	0.195	0.005	0.190
	0.04	0.155	0.015	0.140
	0.08	0.190	0.020	0.170
ML_8	0.01	0.175	0.005	0.170
	0.02	0.210	0.010	0.200
	0.04	0.165	0.020	0.145
	0.08	0.170	0.030	0.140
2:1 AM_2:ML_8	0.01	0.150	0.000	0.150
	0.02	0.170	0.005	0.165
	0.04	0.150	0.012	0.138
	0.08	0.125	0.017	0.108
222	0.01	0.095	0.005	0.090
	0.02	0.100	0.012	0.088
	0.04	0.085	0.015	0.070
	0.08	0.055	0.025	0.030

[a]Control B. stearothermophilus = 0.190.
Control nutrient broth = 0.000.

[b]Difference = A in B.st. - A in control.

[c]Absorbancy at 600 nm in nutrient broth.

Streptococcus lactis strains AM_2, ML_8 did not show nisin production, while a 2:1 (V/V) blend of AM_2:ML_8 strains showed a slight decrease in absorbancy. When 0.08 ml of lactic culture was used as an inoculum, in assay medium, strain 222 decreased absorbancy to 0.030; while the 2:1 (V/V) blend of AM_2:ML_8 strains decreased it only to 0.108 from 0.190, or produced approximately 1/3 nisin. The strains AM_2 and ML_8 independently did not produce nisin at the same extent as the 2:1 (V/V) blend of AM_2:ML_8 strains.

The degree of inhibition (Table 9, row T.S.A.-C; columns B, C, and D) indicated that strain 222 inhibited S. aureus strains at the same extent as the 2:1 (V/V) blend of AM_2:ML_8 strains. Thus, the inhibitory action of the lactic streptococci probably could not be attributed to nisin production.

Evaluation of Inhibitory Response by Starter

Dahiya and Speck (1968) showed that S. aureus MF 31 was inhibited due to the formation of hydrogen peroxide by lactobacilli. They used calcium carbonate fortified trypticase soy agar (T.S.A.-C) to neutralize the effect of acid inhibition and observed that S. aureus was still inhibited. Table 9 indicates the inhibitory response of S. lactis strains AM_2, ML_8 and 2:1 blend of AM_2:ML_8 as well as 222 on T.S.A.-C by spot-tests. The results on trypticase soy agar (T.S.A.) and nutrient agar (N.A.) were a check for inhibition due to acid production.

Table 9. Evaluation of inhibitor production by *S. lactis* AM_2, ML_8 222 and a 2:1 (V/V) blend of AM_2:ML_8 strains by [a] spot tests on trypticase soy agar (T.S.A.), T.S.A. fortified with calcium carbonate (T.S.A.-C.) and nutrient agar (N.A.)

T.S.A.-C.	A[b]	B	C	D
AM_2	-[c]	++	+	++
ML_8	+	++	-	++
2:1	-	++	+	++
222	++	++	++	++
T.S.A.				
AM_2	+	-	++	+
ML_8	-	+	++	+
2:1	-	-	++	+
222	++	+	++	+
N.A.				
AM_2	+ (approx. 30 plaques)	-	++	+
ML_8	-	+	++	+
2:1	-	-	++	+
222	++ (2 plaques)	+	++	+

[a] An 0.03 ml of 8 hr old lactic culture grown at 32 C was spotted on *S. aureus* lawns.

[b] A, B, C and D are enterotoxin-producing strains.

[c] -no inhibition, +inhibition, ++great inhibition.

The results (Table 9) indicated that enterotoxin B and D producing strains were inhibited by both acid and inhibitor but more by the inhibitor. The enterotoxin C producing strain was more acid sensitive, which was also noted by Genigeorgis et al., (1971b), while that producing enterotoxin A behaved in an obscure manner. This strain showed plaques on AM_2 spot in N.A. plates. Though this result could not be reproduced successfully it could be possible that AM_2 strain induces prophage of this lysogenic S. aureus strain. This could be a cause for diminished growth pattern of the S. aureus strain capable of producing enterotoxin A in milk.

Evaluation of Hydrogen Peroxide Production by Starter

Evaluation of hydrogen peroxide production ability by starter was observed in Elliker broth at 32 C for 8 hr. The broth was examined at the end of 4, 6 and 8 hr for the presence of hydrogen peroxide and either it was not produced or was less than 5 µg/ml.

Gilliland and Speck (1969) observed that lactic streptococci produced hydrogen peroxide to a maximum concentration of 0.62 µg/ml. They noted that it was accumulated in early periods of acid production and rapidly dissipated. Hogg and Jago (1970) indicated the importance of an equimolar ratio of thiocyanate to hydrogen peroxide in milk for autoinhibition of lactic streptococci. They also observed approximately 0.6 µg of hydrogen peroxide/ml was necessary

for the autoinhibition of lactic streptococci. They claimed that inhibition was carried out by an oxidized product of thiocyanate. This oxidation reaction was catalyzed by a normal milk enzyme lactoperoxidase in presence of hydrogen peroxide. They also observed the inactivation of hexokinase and aldolase of the lactic streptococci by this inhibitor (cyanosulfuric or cyanosulfurous acid), which resulted in autoinhibition of these bacteria.

Enterotoxigenic staphylococci have been known to produce an exoenzyme catalase which catalyzes the reduction of hydrogen peroxide (Amin and Olsen, 1968). These enzymes, i.e., catalase and lactoperoxidase act on hydrogen peroxide and reduce it. Thus, it could be possible that the inhibition observed in this investigation could be a result of an inhibitor similar to cyanosulfuric or cyanosulfurous acid; and formation of this compound could have been catalyzed by staphylococcal catalase. It can also be postulated that this inhibitor (cyanosulfuric or cyanosulfurous acid) inhibits hexokinase and aldolase of staphylococci more effectively than those of lactic streptococci.

The varied growth inhibition patterns observed for four strains of staphylococci in this investigation also might be correlated to varied catalase activity of enterotoxigenic strains (Amin and Olsen, 1968). This could result in different degrees of inhibitor formation and thereby the differential growth inhibition patterns could be explained.

The inhibition of staphylococci by lactic streptococci, in the present investigation, was not due to hydrogen peroxide.

SUMMARY AND CONCLUSIONS

The effect of reduced levels of starter [2:1 (V/V) blend of AM_2:ML_8 strains of Streptococcus lactis] on the growth of enterotoxins A, B, C and D producing strains of Staphylococcus aureus, was evaluated. The starter used in the present investigation has been consistently used for the manufacture of good-flavored cheddar cheese in New Zealand.

The drop in pH, from 4 to 8 hour incubation, for all starter levels, was proportional to their inocula. The rate of acid formation or, drop in pH, from 4 to 8 hours was correlated with the change in staphylococcal population from 6 to 24 hours (Correlation Coefficient = γ = -0.805). Regression analysis indicated that change in pH from 4 to 8 hours could be used to predict the staphylococcal population change from 6 to 24 hours.

All four enterotoxigenic strains showed differential susceptibility to the starter metabolite(s). A 0.1 per cent starter level did not allow the increase of approximately 10^4 cells per milliliter, of an enterotoxin D producing strain of S. aureus (23235). Approximately 10^6 cells per milliliter of S. aureus 23235, decreased to about 10^4 cells per milliliter in the presence of 0.1 per cent starter level; while 0.01 per cent starter level did not allow the inocula of approximately 10^2 cells per milliliter to increase. The inocula of approximately 10^6 cells per milliliter of

enterotoxin B producing strain of S. aureus, did not increase in the presence of even 0.01 per cent starter level. The same inocula of enterotoxin A and C producing strains of S. aureus decreased to about 10^3 to 10^4 cells per milliliter, respectively, in the presence of 0.01 per cent starter level. These strains sharply declined in population in presence of 0.1 per cent starter level.

The lactic organisms did not produce inhibitory levels nisin, or over 5 micrograms of hydrogen peroxide per milliliter of broth. When the lactic streptococci were spotted on lawns of enterotoxins B, C and D producing strains of S. aureus, staphylococci were inhibited around the spots on both agar with and without added calcium carbonate. Enterotoxin A producing strain was not inhibited on either agar. The degree of inhibition was greater in agar fortified with calcium carbonate for B and D producing strains, while reverse was true for C producing strain.

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